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(54) Title: CYTOKINE ANTIBODY FOR THE TREATMENT OF SEPSIS

(57) Abstract

Compositions and methods for prophylactically or therapeutically treating sepsis consisting of antibody to IL-6 and/or M-CSF wherein the antibodies are administered alone or in combination.

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CYTOKINE ANTIBODY FOR THE TREATMENT OF SEPSIS

This invention is in the area of immunology/biochemistry, and presents cytokine antibody, preferably interleukin 6 (IL-6) and macrophage colony stimulating factor (M-CSF) antibody alone, or in combination, for the prophylactic or therapeutic treatment of sepsis. Antibody may be polyclonal, or monoclonal antibody, or fragments derived therefrom, or recombinant constructs having the binding activity of such antibody.

In the United States alone nosocomial bacteremia develops in about 194,000 patients, and of these about 75,000 die. Maki, D.G., 1981, Nosocomial Infect., (Dikson, R.E., Ed.), page 183, Yrke Medical Books, U.S.A.. Most of these deaths are attributable to six major gram-negative bacilli, and these are Pseudomonas aeruginosa, Escherichia coli, Proteus, Klebsiella, Enterobacter and Serratia. The current treatment for bacteremia is the administration of antibiotics which, unfortunately, have limited effectiveness. Although the precise pathology of bacteremia is not completely elucidated, it is believed that bacterial endotoxins, lipopolysaccharides (LPS), are the primary causative agents. LPS consist of at least three significant antigenic regions, the lipid A, core polysaccharide, and O-specific polysaccharide. The latter is also referred to as O-specific chain or simply O-antigen. The O-specific chain region is a long-chain polysaccharide built up from repeating polysaccharide units. The number of polysaccharide units differs among different bacterial species and may vary from one to as many as six or seven monosaccharide units. While the O-specific chain varies among different gram-negative bacteria, the lipid A and core polysaccharides are similar if not identical.

Since LPS plays a key role in sepsis, a variety of approaches have been pursued to neutralize its activity. Presently, there is considerable work which suggest that antibody to LPS will soon be a valuable clinical adjunct to the standard antibiotic therapy.

LPS initiates a cascade of biochemical events that eventually causes the death of the patient. It is widely believed that the second event, after the introduction of LPS, is the production of tumor necrosis factor (TNF) as a result of LPS stimulation of macrophage cells.

Tumor Necrosis Factor (TNF) is a cytokine which is known to have cytolytic and cytostatic anti-tumor activity. Carswell, et al., 1975, Proc. Nat'l Acad. Sci. USA, 72: 3666-3670; Williamson, et al., 1983, Proc. Nat'l Acad. Sci,. 80: 5397-5401. In addition, it has recently been shown to be a mediator in the immunoinflammatory cascade and play a key role in sepsis. Beutler, et al., 1985, Science, 229: 869,

reported that in a murine model the lethal effect of endotoxin can be reduced by polyclonal rabbit anti-murine TNF antibody. It is likely that antibody to TNF will have valuable clinical applications. Tracey, et al., 1987, Nature, 330:662.

In addition to TNF, another cytokine that is elevated in sepsis patients is interleukin-6 (IL-6). IL-6 is also called hybridoma growth factor, interferon ß-2, B-cell stimulatory factor 2, 26-Kd protein and hepatocyte stimulating factor. The molecule has pleiotropic affects, apparently stimulates hepatic protein synthesis during the acute phase of an infection, and acts as an endogenous pyrogen.

Hack, et al., 1989, Blood, 74:1704, have shown that a significant number of patients with sepsis display increased plasma levels of IL-6, and that the amount of IL-6 correlates with the symptoms of shock and with clinical prognosis. In the sepsis patients shown in that report, serum IL-6 levels were on the order of 1,000 U/ml.

Native IL-6 has a molecular weight of 19-30 kD and an immunoreactive species of 60-70 kD has also been reported (see Kelfgott, et al., 1989, J. Immunol., 142:948 and Jablon, et al., 1989, J. Immunol., 142:1542). The gene coding for a human IL-6 polypeptide has been cloned and expressed as shown by the following European patent applications: EPA 0 220 574, published May 6, 1987, to Revel, M, et al., entitled "Human interferon beta2A and interferon-beta2B, vectors containing genes coding for said interferons, cell lines producing same and use of said interferons as pharmaceuticals"; EPA 0 254 399, published January 27, 1988, to Clevenger, W., et al., entitled "B-cell stimulating factor"; EPA 0 257 406, published March 2, 1988, to Kishimoto, T., et al., entitled "Recombinant B-cell differentiation factor"; EPA 0 261 625, published March 30, 1988, to Honjo, T., et al., entitled "Human B-cell differentiation factor and process of producing said factor"; EPA 0 267 779, published May 18, 1988, entitled "Human pleiotropic immune factor and muteins thereof"; and PCT WO 88/00206, published January 14, 1988, to Clark, S., et al., entitled "Production and use of IL-6".

Antibody to IL-6 has been described both in certain of the foregoing patent applications that show IL-6 (see, for example, EP 257,406), and in the scientific literature.

Another molecule hithertofore unsuspected of being involved in sepsis is macrophage colony stimulating factor (M-CSF), also known as CSF-1. This molecule causes the selective differentiation and proliferation of macrophages, and has been purified from a number of sources. Stanley, E.R., et al., 1977, J Biol Chem, 252:4305 describes the purification from murine L929 cells with a specific activity of about 1 x 10^8 units/mg. Das, S.J., et al., 1982, J Biol Chem., 257:13679 reported that human urinary M-CSF has a specific activity of 5 x 10^7 units/mg and generates

predominately macrophage cells in vitro. Stanley, E.R. and Gilbert, L.J., 1981, Journal of Immunological Methods, 42:253 also describe methods for the purification of M-CSF in low yield. Das, S.K. et al., 1981, Blood, 58:630 describe partial purification of human urinary M-CSF. Wu, N., et al., 1979, J Biol Chem., 254:6226 describe the purification of a CSF that primarily stimulates the formation of macrophages. More recently, M-CSF has been purified in milligram amounts using 10,000 liters of human urine as starting material.

M-CSF has been cloned, and expressed in a number of host cells, and consequently, recombinant M-CSF (rM-CSF) is available for use as an immunogen to elicit antibodies. Indeed, to date, human rM-CSF (hrM-CSF) cDNA clones having three different lengths have been identified, herein denoted α, β and γ. (See Cerretti, et al., 1988, Mol. Immunol, 25:761). They have been isolated from cells expressing the single M-CSF gene. The α, β and γ clones contain M-CSF DNA sequences that encode unprocessed proteins having 224, 522 and 438 amino acids, respectively.

Recombinant M-CSFs have been expressed in active form, and thus these molecules may be used to generate suitable monoclonal antibodies. The preferred rM-CSF is that described in U.S. Patent No. 4,847,201, to E. Kawasaki et al., issued July 11, 1989. Therein is shown the expression, in both prokaryotes and eukaryotes, of the α form of M-CSF.

In addition to being defined by their biological activities, M-CSF and IL-6 may also be defined by their chemical structures. The DNA and amino acid sequences of IL-6 are known. In contrast, the precise structure of naturally produced M-CSF is not clearly apparent from a reading of the scientific literature. For instance, human M-CSF purified from urine is thought to consist of two essentially identical subunits with an apparent molecular weight of 25-35 kilodaltons. M-CSF purified from a pancreatic carcinoma cell line, MIA PaCa-2, was reported to consist of two subunits, but with apparent molecular weights of about 23-100 kilodaltons. These differences may be due to differences in glycosylation, or may arise as a result of alternative splicing of the M-CSF mRNA transcript, as described above. See also Stradle, et al., 1989, J. Cell. Biochem., 40:91. The M-CSF proteins precursor from the ß clone is thought to give rise to a 70-90 kilodalton glycoprotein, believed to be a dimer with 35-45 kilodalton subunits, possibly having about 223-224 amino acids. The smaller precursor yields a 40-50 kilodalton glycoprotein, also comprising a dimer with a subunit molecular weight of about 20-25 kilodaltons, and possibly with about 158 amino acids. See Halenbeck, R., et al., 1988, Biotechnology J., 8:45. Thus, it should be apparent that within the structural definition of M-CSF there exists a set of related proteins of varying molecular

weights. It should be further apparent from the foregoing discussion that the definition of M-CSF is not restricted to proteins with the above-described molecular weights. It is to be anticipated, in light of the existence of multiple mRNAs coding for M-CSF, that proteins with molecular weights different from those discussed above will be discovered, and thus are intended to come within the definition of M-CSF.

It will further be appreciated with regard to M-CSF or IL-6, that their precise structure depends on a number of factors. As all proteins contain ionizable amino and carboxyl groups it is, of course, apparent that they may be obtained in acidic or basic salt form, or in neutral form. It is further apparent, that the primary amino acid sequence may be augmented by derivatization using sugar molecules (glycosylation) or by other chemical derivatizations involving covalent, or ionic attachment of, for example, lipids, phosphate, acetyl groups and the like, often occurring through association with saccharides. These modifications may occur in vitro, or in vivo, the latter being performed by a host cell through post-translational processing systems. It will be understood that such modifications, regardless of how they occur, are intended to come within the definition of M-CSF, and IL-6 so long as the activity of the protein, as defined above, is not destroyed. It is to be expected, of course, that such modifications may quantitatively or qualitatively increase or decrease the biological activity of the molecule, and such chemically modified molecules are also intended to come within the scope of the definition of M-CSF, and Il-6 since these molecules would be expected to elicit medically useful antibodies.

A first object of the invention is a description of cytokine antibody, preferably IL-6 and/ or M-CSF antibody for the prophylactic or therapeutic treatment of sepsis.

A second object of the invention is a description of IL-6 and/or M-CSF antibody, or antibody fragments derived therefrom, for the prophylactic or therapeutic treatment of sepsis wherein the antibody is polyclonal, monoclonal, or recombinant constructs having the binding activity of such antibody or antibody fragments.

A third object of the invention is a description of a mixture of antibodies for the prophylactic or therapeutic treatment of sepsis consisting of IL-6 and M-CSF antibody, antibody fragments derived therefrom, or recombinant constructs having the binding activity of such antibody or antibody fragments.

A fourth object of the invention is a description of methods for administrating IL-6 and/or M-CSF antibody for the prophylactic or therapeutic treatment of sepsis.

These, and other objects of the invention, will be more fully understood after a consideration of the following description of the invention.

Figure 1 shows IL-6 levels in baboon plasma after a lethal or sublethal dose of E. coli.

Figure 2 shows that IL-6 monoclonal antibody considerably extends the lifetime of a baboon administered a lethal dose of E. coli.

Figure 3 shows the effect of administering IL-6 monoclonal antibody, 8M70, to a baboon on various physiologically parameters prior to the baboon receiving a lethal dose of E. coli.

Figure 4 shows an increase in M-CSF levels in baboons administered a lethal dose of E. coli.

The present invention is directed to the production and utilization of cytokine antibody, preferably IL-6 and/or M-CSF antibody for the prophylactic or therapeutic treatment of sepsis. Several patents/patent applications and scientific references are referred to below that discuss various aspects of the material and methods used to realize the invention. Because the invention draws on these materials and methods, it is thus intended that all of the references, in their entirety, be incorporated by reference.

To more clearly define the present invention, particular terms herein will be employed according to the following definitions generally consistent with their usage in the art.

"Sepsis" is herein defined to mean a disease resulting from bacterial infection due to the bacterial endotoxin, lipopolysaccharide (LPS). It can be induced by at least the six major gram-negative bacilli and these are Pseudomonas aeruginosa, Escherichia coli, Proteus, Klebsiella, Enterobacter and Serratia. It is expected that sepsis induced by gram-positive organisms may also be beneficially treated with the approaches described herein.

"Monoclonal antibody" refers to a composition of antibodies produced by a clonal population (or clone) derived through mitosis from a single antibody-producing cell. A composition of monoclonal antibodies is "substantially free of other antibodies" when it is substantially free of antibodies that are not produced by cells from the clonal population. The term "substantially free" means approximately 5% (w/w) or fewer contaminating antibodies in the composition. Also intended to come within the scope of the definition are modifications to antibody that increase its effectiveness. A preferred modification includes conjugation of a water soluble polymer. Preferably the water soluble polymer is polyethylene glycol, or a functionally related molecule such as, for example, polypropylene glycol homopolymers, polyoxyethylated polyols, and polyvinyl alcohol. Derivatization of antibody with such water soluble polymers increases its in vivo half-life, reduces its immunogenicity, and reduces or eliminates aggregation of the protein and may reduce its immunogenicity and aggregation that might occur when it is introduced in vivo. Derivatization of proteins generally, or antibody specifically, with water soluble polymers such as those described above are

presented in U. S. Patent Nos. 4,179,337, issued December 18, 1979, to Davis et al., entitled "Non-immunogenic polypeptides"; and 4,732,863, issued March 22, 1988, to Tomasi, et al., entitled "PEG-modified antibody with reduced affinity for cell surface Fc receptors", respectively.

An "antibody-producing cell line" is a clonal population or clone derived through mitosis of a single antibody-producing cell capable of stable growth in vitro for many generations.

"Tumor Necrosis Factor" or "TNF" as used herein refers to both native and recombinant forms of this known, mammalian cytokine. TNF has been referred to by other names in the literature, including "Cachectin" and "TNF-α". "Recombinant TNF" or "rTNF" refers to proteins, including muteins, produced by expression of recombinant DNA that have the same or substantially the same amino acid sequence as native TNF (or portions thereof), and retain both the in vitro and in vivo biological activity of TNF. The isolation and production of both native and recombinant mammalian TNF, including human TNF, is known in the art. See, e.g., Carswell et al., 1975, Proc. Nat'l Acad. Sci. USA, 72: 3666-3670; Williamson et al., 1983, Proc. Nat'l Acad. Sci. USA, 80: 5397-5401; Wang et al., 1985, Science, 228:149-154; Beutler et al., 1985, Science, 229: 869; Beutler et al., 1985, Nature, 316: 552; Pennicia et al., 1984, Nature, 312:724; Aggarwal et al., 1985, J. Biol. Chem., 260: 2345.

"Recombinant antibody" refers to antibody wherein one portion of each of the amino acid sequences of heavy and light chain is homologous to corresponding sequences in antibody derived from a particular species or belonging to a particular class, while the remaining segment of the chains is homologous to corresponding sequences in another. Most commonly, in a recombinant antibody the variable region of both light and heavy chain mirrors the variable regions of antibody derived from one species of mammals, while the constant regions are homologous to the sequences in antibody derived from another. However, this is not necessarily always the case; for example, Ward, et al., 1989, Nature, 341:544, have shown that variable chain alone can be expressed in bacteria with significant antigen binding activity.

Two antibodies are "cross-blocking" or have a "shared epitope" when each antibody effectively blocks the binding of the other antibody in a binding inhibition assay. Thus, if antibodies A and B are cross-blocking, antibody A would not bind to its antigen when the antigen had been preincubated with antibody B, and antibody B would not bind to its antigen when the antigen had been preincubated with antibody A.

The term "binding affinity" or "Ka" of an antibody to its epitope, as used herein, refers to a binding affinity calculated according to standard methods by the

formula Ka = 8/3(It-Tt), where It is the total molar concentration of inhibitor uptake a 50% tracer, and Tt is the total molar concentration of tracer. See Muller, 1980, J. Immunol. Methods, 34: 345-352.

As used herein, the term "incubation" means contacting antibodies and antigens under conditions that allow for the formation of antigen/antibody complexes (e.g., proper pH, temperature, time, medium, etc.). Also as used herein, "separating" refers to any method, usually washing, of separating a composition from a test support or immobilized antibody, such that any unbound antigen or antibody in the composition are removed and any antigen/antibody complexes on the support remain intact. The selection of the appropriate incubation and separation techniques is within the skill of the art.

I. IL-6/M-CSF Antibody

In a preferred embodiment of the invention, IL-6 or M-CSF antibody producing immunologic cells are isolated from a mammal immunized with IL-6 and/or M-CSF, and immortalized to yield antibody secreting cell lines e.g. hybridomas, triomas etc. Cell lines that secrete the desired antibody can be identified by assaying culture supernatants for antibody activity. Thus, the invention can be broken down into three sections, and each section discussed separately. That is, the immunization procedure, the cell immortalization procedure, and the identification of IL-6 and/or M-CSF antibody.

A. Immunization with IL-6/M-CSF

IL-6 and M-CSF, alone or in combination, may be used to immunize an appropriate host animal. Preferably, the host animal is immunized with IL-6 described by Brakenhoff et al., 1987, Journal of Immunology, 139:4116, or in EP 257,406, or M-CSF described in U.S. Patent No. 4,847,201. A suitable adjuvant may be used to enhance the immune response. A variety of distinguishable immunization protocols may be employed, and may consist of a primary intravenous, subcutaneous, or intraperitoneal immunization followed by one or more boosts. The precise immunization schedule is generally not critical, and determinative of which procedure is employed, is the presence of M-CSF or IL-6 antibody in the host animal as measured by a suitable assay, described below.

Alternatively, lymphocytes may be immunized in vitro. For example, immunization of peripheral blood cells may be achieved as described by Boss, Methods of Enzymology, 121(I), and in EPA 86106791.6. Note particularly in vitro immunization techniques that can be used to produced either murine or human

monoclonal (Procedures for Transforming Cells, pages 18-32, 140-174, Methods of Enzymology, vol.121, part I). Such techniques are also described by Luben, R. and Mohler, M., 1980, Molecular Immunology, 17:635, Reading, C. Methods in Enzymology, 121 (Part One):18, or Voss, B., 1986, Methods in Enzymology, 121:27. A number of in vitro immunization systems have been shown to be effective for sensitizing human B-cells. Reading, C., 1982, J. of Immun. Methods, 53:261.

It will be apparent to those skilled in the art, that in lieu of immunizing individuals directly with IL-6 or M-CSF, lymphocytes may be isolated from individuals that are experiencing, or have experienced a bacteremic attack. A fraction of these lymphocytes may be sensitized to these molecules and can be used to produce permanent antibody secreting hybrid cell lines. For example, immunocompromised human patients are generally susceptible to bacterial infections, particularly those suffering from various malignancies, extensive burns, etc., and lymphocytes isolated therefrom may be a source of antibody secreting cells.

In lieu of using IL-6 and M-CSF as immunogens, an alternative approach is to synthesize IL-6 or M-CSF peptides, and use these as immunogens. For example, a particularly useful peptide to produce antibody that binds to IL-6 is described in Japanese patent application No. 62102157. The methods for making antibody to peptides are well known in the art and generally require coupling the peptides to a suitable carrier molecule, such as serum albumin. The peptides can be made by techniques well known in the art, such as, for example, the Merrifield solid-phase method described in Science, 232:341-347 (1985). The procedure may use commercially available synthesizers such as a Biosearch 9500 automated peptide machine, with cleavage of the blocked amino acids being achieved with hydrogen fluoride, and the peptides purified by preparative HPLC using a Waters Delta Prep 3000 instrument, on a 15-20 μm Vydac C4 PrepPAK column. Once clones are identified that secrete anti-peptide antibody, the antibody can be screened for binding and neutralizing activity to either IL-6 or M-CSF.

B. IL-6/M-CSF Antibody

Antibody to IL-6 or M-CSF may be either polyclonal, monoclonal, or fragments derived therefrom . The antibody is preferably human or humanized, although non-human antibody will perform satisfactory. Additionally, recombinant constructs having the antibody binding specificity of antibody to IL-6 or M-CSF may be produced.

The preparation of high-titer neutralizing polyclonal antibody can be realized by immunizing a variety of species and employing one of several different immunization regimes. The preferred method of the instant invention is to immunize rabbits with IL-6 or M-CSF prepared in complete Freund's adjuvant by injection into axial lymph nodes. The animals are subsequently subjected to multiple boosts (containing about half the original amount of IL-6 or M-CSF) in incomplete Freunds adjuvant at about 21-day intervals. About 10 days following each 21-day interval, 20-30 ml of blood is removed, the serum isolated and antibody isolated therefrom. This procedure may be carried out for a period of several months.

Monoclonal antibody may be produced using IL-6, M-CSF, or peptides/peptide conjugates of these molecules as described above, and using the procedures described by Kohler, G. and Milstein, C., 1975, Nature, 256:495, or modifications thereof that are known in the art. Using the screening assays described below, the specificity of antibody produced can be discerned.

The initial work of Kohler and Milstein, above, involved fusing murine lymphocytes and drug selectable plasmacytomas to produce hybridomas. Subsequently, the technique has been applied to produce hybrid cell lines that secrete human monoclonal antibodies. The latter procedures are generally described in Abrams, P., 1986, Methods in Enzymology, 121:107, but other modifications are known to those skilled in the art. Regardless of whether murine or human antibody is produced, the antibody secreting cells are combined with the fusion partner and the cells fused with a suitable fusing agent, preferably polyethylene glycol, and more preferably polyethylene glycol 1000. The latter is added to a cell pellet containing the antibody secreting cells and the fusion partner in small amounts over a short period of time accompanied with gentle agitation. After the addition of the fusing agent, the cell mixture is washed to remove the fusing agent and any cellular debris, and the cell mixture consisting of fused and unfused cells seeded into appropriate cell culture chambers containing selective growth media. After a period of several weeks, hybrid cells are apparent, and may be identified as to antibody production and subcloned to ensure the availability of a stable hybrid cell line.

The preferred antibody is human monoclonal antibody which can be prepared from lymphocytes sensitized with IL-6/M-CSF either in vivo or in vitro by immortalization of antibody-producing hybrid cell lines, thereby making available a permanent source of the desired antibody, using the cell fusion techniques described above. Alternatively, sensitized lymphocytes may be immortalized by a combination of two techniques, viral transformation and cell fusion. The preferred combination consist of transforming antibody secreting cells with Epstein-barr virus, and subsequently

fusing the transformed cells to a suitable fusion partner. Such fusion partners are known in the art, and exemplary partners may be a mouse myeloma cell line, a heteromyeloma line, or a human myeloma line, or other immortalized cell line. PCT Patent Application No. 81/00957; Schlom et al., 1980, PNAS USA, 77:6841; Croce et al., 1980, Nature, 288:488. The preferred fusion partner is a mouse-human hetero-hybrid, and more preferred is the cell line designated F3B6. This cell line is on deposit with the American Type Culture Collection, Accession No. HB8785. It was deposited April 18, 1985. The procedures for generating F3B6 are described in European Patent Application, Publication No. 174,204.

Techniques applicable to the use of Epstein-Barr virus transformation and the production of immortal antibody secreting cell lines are presented by Roder, J. et al., 1986, Methods in Enzymology, 121:140. Basically, the procedure consist of isolating Epstein-Barr virus from a suitable source, generally an infected cell line, and exposing the target antibody secreting cells to supernatants containing the virus. The cells are washed, and cultured in an appropriate cell culture medium. Subsequently, virally transformed cells present in the cell culture can be identified by the presence of the Epstein-Barr viral nuclear antigen, and transformed antibody secreting cells can be identified using standard methods known in the art.

It will be apparent to those skilled in the art, and as mentioned above, that while the preferred embodiment of the instant invention is neutralizing Il-6 or M-CSF monoclonal antibody, singly or in combination, that the antibody(s) may be altered and still maintain biological activity. Thus, encompassed within the scope of the invention is antibody modified by reduction to various size fragments, such as $F(ab')_2$, Fab, Fv, or the like. Also, the hybrid cell lines that produce the antibody may be considered to be a source of the DNA that encodes the desired antibody, which may be isolated and transferred to cells by known genetic techniques to produce genetically engineered antibody. An example of the latter would be the production of single chain antibody having the antibody combining site of the hybridomas described herein. Single chain antibody is described in U.S. Patent No. 4,704,692.

A second example of genetically engineered antibody is recombinant, or chimeric antibody. Methods for producing recombinant antibody are shown in U.S. Patent No. 4,816,567, to Cabilly, et al.; Japanese patent application, Serial No. 84169370, filed August 15, 1984; British patent application 8422238, filed on September 3, 1984; and Japanese patent application, No. 85239543, filed October 28, 1985. Also, British patent application, No. 867679, filed March 27, 1986, describes methods for producing an altered antibody in which at least parts of the complementary determining regions (CDRs) in the light or heavy chain variable domains have been

replaced by analogous parts of CDRs from an antibody of different specificity. Using the procedures described therein it is feasible to construct recombinant antibody having the CDR region of one species grafted onto antibody from a second species that has its CDR region replaced.

Regardless of the type of antibody, polyclonal or monoclonal etc., it is desirable to purify the antibody by standard techniques as is known in the art, or as described by Springer, 1980, Monoclonal Antibodies.:194, (Eds. Kennett, T. McKearn and K. Bechtol, Plenum Press, New York. Generally this consists of at least one ammonium sulfate precipitation of the antibody using a 50% ammonium sulfate solution. Antibody affinity columns may also be used.

The preferred IL-6 antibody is denoted 8M70, and methods for obtaining it are described below in the Example section.

C. Screening of IL-6/M-CSF Antibody

Cell lines that secrete IL-6 or M-CSF antibody can be identified by assaying culture supernatants, ascites fluid etc., for antibody. The preferred screening procedure consists of two sequential steps. First, hybridomas are identified that secrete antibody; and second, the antibody is assayed to determine if it exhibits neutralizing activity.

As applied to cell culture supernatants, the initial screening step is preferably done by RIA or ELISA assay. Both assays are known in the art, and consists of binding IL-6 or M-CSF to a solid matrix, and assaying for antibody binding to these molecules as revealed by a second, labelled antibody. For as description of the ELISA assay method see Langone, J. and Van Vinakis, H., 1983, Methods of Enzymology, 92, Part E, and for a description of RIA assay see Miller et al., 1983, Method in Enzym., 121:433 Part I. If peptides are used as immunogen, the initial screening step determines if the antibody binds to peptide conjugates bound to a solid matrix.

An additional assay for IL-6 or M-CSF antibody is to screen for antibody that immunoprecipitates IL-6 or M-CSF from solution. For example, supernatants being tested for the presence of antibody may be incubated with labelled IL-6 or M-CSF for an appropriate time to allow antigen/antibody complexes to form. The complex may be washed to removed any unreacted reagents, and next the antibody complexes incubated with anti-xenotypic or anti-isotypic antibodies specific for the monoclonal antibody being screened. These anti-xenotypic or anti-isotypic antibodies may be immobilized, for example, on a plastic bead. Thus, if the monoclonal antibody being screened is IL-6 antibody, then labelled IL-6 will be indirectly bound to the bead and thereby immunoprecipitated. IL-6 antibody can then be quantitated using suitable detection methods known in the art dependent on the nature of the label used. Also, the material

can be dissociated from the bead using standard techniques and identified by gel electrophoresis, as is known in the art.

The preferred electrophoresis procedure is Western Blot gel analysis as described by Burnette, 1981, Anal. Bio. Chem., 112:195. The Western blots are blocked, washed, and probed preferably in 10 mM sodium phosphate buffer containing 150 mM sodium chloride (pH 7.4), with 0.1% bovine serum albumin (w/v), and 0.1% ovalbumin (w/v). In addition, a detergent is preferably employed such as Tween 20 at a concentration of about 0.1%. Sodium azide may also be included in the solution at a concentration of 0.02%. The blots are preferably first probed with either hybridoma culture supernatant, or dilute ascites fluid containing IL-6 or M-CSF antibody, washed, and then antibody binding revealed with ^{125}I-protein A for about 30-60 minutes. The blots are washed, and subjected to autoradiography using X-ray film.

To expedite the time it takes to assay for IL-6 and M-CSF antibody, several culture supernatants may be combined and assayed simultaneously. If the mixture is positive, then media from each well may subsequently be assayed independently to confirm the presence of antibody.

II. Immunoassay

In another embodiment, the present invention is directed to an immunoassay which can be used to detect IL-6 and/or M-CSF levels which are indicative of the prognosis of sepsis patients. The concentrations that are detectable will be in the range of less than 1 ng/ml to about 1 ug/ml of either molecule. The immunoassay of the present invention is preferably a sandwich assay employing the antibodies disclosed herein, although other assay formats known in the art may also be used.

In practicing the immunoassay method of the present invention, labelled IL-6 or M-CSF antibody is incubated with the fluid test sample taken from a patient containing unknown concentrations of these molecules. After allowing for a suitable period of incubation for antigen-antibody complexes to form, the mixture is washed and incubated with an indicator solution containing a solid matrix to which is bound antibody that binds IL-6 and/or M-CSF antibody, and such antibody may be either monoclonal or polyclonal. This second antibody is incubated for a sufficient period of time to allow antigen-antibody complexes to form between the labeled antibody and/IL-6 and/or M-CSF. After this incubation, the immobilized complex is separated from any unbound reactants, and the amount of label bound to the immobilized antibody is measured. (See, for example, Shadle, et al., 1989, Exp. Hematol., 17:154).

It will be appreciated by those skilled in the art that while IL-6 and M-CSF may be measured independently in different sample aliquots, that they may also be measured simultaneously in the same fluid. If they are measured simultaneously, the same general procedures as described above may be followed and further incorporating technical means whereby the concentrations of IL-6 and M-CSF can be distinguished. Such means are well known in the art and may consists of differentially labelling IL-6 and M-CSF antibody.

The antibodies employed in the present invention can be immobilized on any appropriate solid test support by any appropriate technique. The solid test support can be any suitable insoluble carrier material for the binding of antibodies and immunoassays. Many such materials are known in the art, including, but not limited to, nitrocellulose sheets or filters; agarose, resin, plastic (e.g. PVC or polystyrene) latex, or metal beads; plastic vessels; and the like. Many methods of immobilizing antibodies are also known in the art. See, e.g., Silman et al., 1966, Ann. Rev. Biochem., 35: 873; Melrose, 1971, Rev. Pure & App. Chem., 21: 83; Cuatrecaas et al., 1971, Meth. Enzym., 22. Such methods include covalent coupling, direct adsorption, physical entrapment, and attachment to a protein-coated surface. In the latter method, the surface is first coated with a water-insoluble protein such as zein, collagen, fibrinogen, keratin, glutelin, etc. The antibody is attached by simply contacting the protein-coated surface with an aqueous solution of the antibody and allowing it to dry.

Any combination of support and binding technique which leaves the antibody immunoreactive, yet sufficiently immobilizes the antibody so that it can be retained with any bound antigen during a washing, can be employed in the present invention. A preferred solid test support is a plastic bead.

As discussed above, the assay of the present invention employs a labelled antibody. The label can be any type that allows for the detection of the antibody when bound to a support. Generally, the label directly or indirectly results in a signal which is measurable and related to the amount of label present in the sample. For example, directly measurable labels can include radio-labels (e.g. 125I, 35S, 14C, etc.). A preferred directly measurable label is an enzyme, conjugated to the antibody, which produces a color reaction in the presence of the appropriate substrate. (e.g. horseradish peroxidase/o-phenylenediamine). An example of an indirectly measurable label is antibody that has been biotinylated. The presence of this label is measured by contacting it with a solution containing a labeled avidin complex, whereby the avidin becomes bound to the biotinylated antibody. The label associated with the avidin is then measured. A preferred example of an indirect label is the avidin/biotin system

employing an enzyme conjugated to avidin, the enzyme producing a color reaction as described above.

Whatever label is selected, it results in a signal which can be measured and is related to the amount of label in a sample. Common signals are radiation levels (when radioisotopes are used), optical density (e.g. when enzyme color reactions are used) and fluorescence (when fluorescent compounds are used). It is preferred to employ a nonradioactive signal, such as optical density (or color intensity) produced by an enzyme reaction. Numerous enzyme/substrated combinations are known in the immunoassay art which can produce a suitable signal. See, e.g., U.S. Patent Nos. 4,323,647 and 4,190,496, the disclosures of which are incorporated herein.

III. Treatment of Sepsis

The IL-6 or M-CSF antibodies described herein, alone or in combination, may be used to passively immunize a host organism suffering from bacteremia or sepsis, or at risk with respect to bacterial infection. Either anti-IL-6 alone, anti-M-CSF along, or preferably anti-IL-6 and anti-M-CSF will be administered. Treatment will generally consist of administering the antibodies parenterally, and preferably intravenously. The dose and administration regime will be a function of whether the antibodies are being administered therapeutically or prophylactically, and the patient's medical history. Typically, the amount of antibody administered per dose will be in the range of about 0.1 to 25 mg/kg of body weight, with the preferred dose being about 0.1 to 10 mg/kg of patient body weight. For parenteral administration, the antibodies will be formulated in an injectable form combined with a pharmaceutically acceptable parenteral vehicle. Such vehicles are well known in the art and examples include water, saline, Ringer's solution, dextrose solution, and solutions consisting of small amounts of the human serum albumin. The vehicle may contain minor amounts of additives that maintain the isotonicity and stability of the antibody. The preparation of such solutions is within the skill of the art. Typically, the antibodies will be formulated in such vehicles at a concentration of about 2-8.0 mg/ml to about 100 mg/ml.

The effectiveness of the subject IL-6 and M-CSF antibodies in the treatment of sepsis an be demonstrated in one of several animal model systems. The preferred animal model system is baboon, and is described by Taylor, et al., 1987, J. of Clinical Inv., 79:918, and by Taylor, et al., 1988, Circulatory Shock, 26:227. Briefly, this consist of infusing a lethal dose of E. coli, about 4 x 10^{10} organisms per kilogram of body weight administered over a 2-hour period. This is sufficient to kill 100% of the test animals in a period ranging from 16-32 hours. The animals are anesthetized with sodium pentobarbital in the cephalic vein through a percutaneous catheter. They are

also orally intubated and positioned on their right side on a heating pad. Blood samples are removed from the femoral vein which is aseptically cannulated in the hind limb. The percutaneous catheter is used to infuse the E. coli organisms. Blood samples are taken at desired time intervals and assayed for white blood cells hematocrit, platelet levels, and fibrinogen. Additionally, mean systemic arterial pressure (MSAP) may be monitored with a transducer (Stratham P2306, Porter) pressure gauge. Changes in these parameters may be prognostic of a patients ability to withstand exposure to a lethal dose of bacteria.

Having described what the applicants believe their invention to be, the following examples are presented to illustrate the invention, and are not to be construed as limiting the scope of the invention. For example, variation in the source, type, or method of producing antibodies; different labels and/or signals; test supports of different materials and configurations; different immobilization methods may be employed without departing from the scope of the present invention.

Example I

Preparation of IL-6, M-CSF or IL-6, M-CSF Peptide Immunogens

A. IL-6, M-CSF

IL-6 was prepared as described by Brakenhoff et al., 1987, Journal of Immunology, 139:4116. Alternative methods are shown in EPC patent application publication no. 261,625 and PCT patent application international publication number W0 88/00206. Recombinant M-CSF was produced as described in U.S. Patent No. 4,847,201. The latter patent describes the expression of monomeric M-CSF which, in the mammalian expression system shown, spontaneously recombines and refolds to yield biologically active M-CSF dimers. Preferably, in lieu of using spontaneously refold M-CSF, the monomers may be refolded as described in PCT patent application, International Publication No. WO88/08003. Additionally, M-CSF may be produced using the methods described in U.S. Patent Nos. 4,868,119, and 4,879,227.

B. Peptide Conjugates

Based on the known amino acid sequences of IL-6 and M-CSF, peptides are synthesized and tested for immunogenic activity, binding to IL-6 and M-CSF, and neutralization of the biological activity of these molecules. Peptides may be synthesized using the solid-phase method, described in detail in Merrifield R.B., 1985, Sci., 232:341-347, on a Biosearch 9500 automated peptide machine, cleaved with hydrogen fluoride, and purified by preparative HPLC using a Waters Delta Prep 3000 instrument, on a 15-20 μm Vydac C4 PrepPAK column. The preferred peptide for producing

antibody to IL-6 is described in Japanese Patent No. 62102157 and has the amino acid sequence: Pro-Val-Pro-Pro-Gly-Glu-Asp-Ser-Lys-Asp-Val-Ala-Ala.

M-CSF peptides that may be employed are those taken from amino acids 4-150 of the mature molecule. That is, M-CSF which lacks the leader sequence. Exemplary peptides include; Thr-Ala-Pro-Gly-Ala-Ala-Gly-Arg-Cys-Pro-Pro-Thr, and Met-Ile-Gly-Ser-Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser.

Before using the peptides to make antibody they are conjugated to a suitable carrier molecule to enhance eliciting an antibody response. These procedures are described in U.S. Patent No. 4,762,706, inventors McCormick, et al.. Suitable carriers are keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA). The conjugation is achieved via a sulfhydryl group of a cysteine residue that, if necessary, is added to the amino or carboxyl terminal end of the peptides. A heterobifunctional crosslinking reagent, N-maleimido-6-amino caproyl ester of 1-hydroxy-2-nitro-benzene-4-sulfonic acid sodium salt, is prepared by the following procedure.

One molar equivalent (2.24 g) of 4-hydroxy-3-nitro-benzene sulfonic acid sodium salt (HNSA) is mixed together with one molar equivalent (2.06 g) of dicyclohexylcarbodiimide and one molar equivalent (2.10 g) of N-maleimido-6-aminocaproic acid in 25 ml of dimethylformamide (DMF) at room temperature overnight. A white precipitate of dicyclohexyl urea is formed. The precipitate is filtered and 300 ml diethyl ether is added to the mother liquor. After about 10 minutes to 4 hours a gummy solid precipitated from the mother liquor is formed. This solid will contain 58% of active HNSA ester and 42% of free HNSA.

The analysis consists of dissolving a small amount of the precipitate in phosphate buffer at pH 7.0 and measuring the absorbance at 406 nm; this reading provides the amount of unreacted free HNSA which is the contaminating material in the HNSA ester preparation. Addition of very small amounts of concentrated strong base (such as 5N NaOH) instantly hydrolyses the ester formed and a second reading is taken. Subtraction of the first reading from the second yielded the amount of ester in the original material. The solid is then dissolved in DMF and placed on a LH20 Sephadex column and eluted with DMF so that the ester is separated from the contaminating free HNSA. The progress of purification is monitored by thin layer chromatography using eluting solvents of chloroform, acetone and acetic acid (6:3:1 vol/vol). The product is positively identified as mal-sac HNSA ester by its reactivity with amine. The yield of the pure ester is estimated to be approximately 30% of theoretical; the purified material consists of 99% ester.

The ester thus obtained is found to dissolve fully in water and is stable in water for several hours, provided no nucleophiles are added. When placed in 1N ammonia

the ester produces the corresponding amide with a portion hydrolyzed to free acid. The purified ester is found to be stable for extended periods when stored dessicated.

About 0.5 mg of the purified mal-sac HNSA ester is dissolved in 1 ml of distilled water. A 10 µl aliquot of this solution is diluted into 1 ml of 10 mM phosphate buffer at pH 7.0. The absorbance at 406 nm is used to calculate the concentration of free HNSA as described above. When 50 µl of 4.8N sodium hydroxide solution is added to the diluted aliquot of ester and mixed, the absorbance of the solution at 406 nm increases significantly, indicating that the hydroxide nucleophile rapidly hydrolyses the ester to component acid and free HNSA anion.

The difference between the post-base and initial free HNSA concentration represents the concentration of ester. From the actual concentration of ester and protein amino groups the amount of ester to be added to the protein solution to achieve the desired degree of substitution can be calculated.

The purified HNSA ester is then reacted with BSA as follows (the reaction with KLH is similar to this procedure):

A total of 22 mg (20 µmoles) of BSA (of molecular weight 66,296) is dissolved in 2.0 ml of 0.1 M phosphate buffer at pH 7.5 to yield a total amine concentration of 1.0×10^{-2} moles per liter (assuming 59 lysines/BSA molecule). A calculated amount (11 mg, 2.35×10^{-5} moles) of the above-prepared mal-sac HNSA ester (97.7% pure) in powder form is dissolved in 2.0 ml of BSA solution. The reaction is carried out at room temperature. Ten µl aliquots are removed from the solution at timed intervals and are each diluted into 1.0 ml of 0.01 M phosphate buffer at pH 7.0. The spectrum of each diluted aliquot is recorded using a Hewlett-Packard spectrophotometer and the absorbance at 406 nm measured. A total of 50 µl of 4.8N NaOH is then added to each aliquot, each aliquot is mixed and its spectrum retaken, and the absorbance at 406 nm measured.

From the absorbance at 406 nm before and after addition of base the concentration of ester remaining and the percent ester that reacts are determined for the reaction mixtures. The results show that the reaction rate is essentially linear over a 15-minute period.

After 15 minutes of reaction time, the reaction is stopped by applying the reaction mixture to a PD10 desalting Sephadex G-25 column (Pharmacia, Inc.) equilibrated with 0.1 M phosphate buffer at pH 6.0. It is found that 2.6 x 10-3 moles/l of the ester reacts, and thus 25.9% of the 59 epsilon-amino groups of BSA are presumably substituted. Thus, the product contains 16 mal-sac groups per molecule.

The product of the first reaction, mal-sac-BSA (or mal-sac-KLH), is isolated by applying the reaction mixture to a PD10 desalting Sephadex G-25 column equilibrated

with 0.1 M phosphate buffer at pH 6.0. The column is eluted with 0.1 M phosphate buffer in 1.0 ml fractions. The column elution is followed by monitoring the absorbance spectrum, and peak fractions containing the mal-sac BSA are pooled.

The peptides synthesized as described above are added and the pooled mixture is stirred at room temperature overnight. The conjugates are subjected to extensive dialysis against distilled water and lyophilization, and in some cases are analyzed for changes in amino acid composition. These peptide conjugates may be used to immunize animals, or lymphocytes in vitro to produce the desired antibody.

Example II

Immunization with IL-6, M-CSF or Peptide Immunogens and the Production of Hybridomas

A. Murine Monoclonal Antibody

The following describes the immunization of mice with M-CSF with the aim of isolating immunized lymphocytes and producing murine hybridomas. This procedure is also applicable to generating antibody against IL-6. It will be further appreciated that the procedure can be employed to produce antibody against M-CSF, or IL-6, or M-CSF peptides, synthesized and conjugated as described above.

Generally, the procedures described in the following references are followed for generating hybridomas. Shulman et al., 1978, Nature, 276:269; Oi et al., in Selected Methods in Cellular Immunology, p 351 (Mischell & Schiigi eds. 1980). Foung et al., 1983, Proc. Nat'l Acad. Sci. USA, 79:7484. Further references include, Gerhard et al., 1978, Proc. Nat'l Acad. Sci.USA, 75: 1510; Monoclonal Antibodies (R. Kennett, T. McKearn, & K. Bechtol eds. 1980); Schreier et al., 1980, Hybridoma Techniques; Monoclonal Antibodies and T-Cell Hybridomas (G. Hammerling, U. Hammerling, & J. Kearney eds. 1981); Kozbor et al., 1982, Proc. Nat'l Acad. Sci. USA, 79: 6651; Jonak et al., 1983, Hybridoma, 2: 124; Monoclonal Antibodies and Functional Cell Lines (R. Kennett, K. Bechtol, & T. McKearn eds. 1983); Kozbor et al., 1983, Immunology Today, 4:72-79; Shulman et al., 1982, Nature, 276: 269-270; Oi et al., in Selected Methods and Cellular Immunology, pp. 351-371 (B. Mischell & S. Schiigi eds. 1980); Foung et al., 1983, Proc. Nat'l Acad. Sci. USA, 79:7484-7488.

Balb/c mice were immunized with recombinant M-CSF (rM-CSF). Immunization consisted of a primary intraperitoneal immunization of 40 µg of rM-CSF in complete Freunds adjuvant, followed by two subsequent intraperitoneal injections without complete Freunds adjuvant, consisting of 20 µg rM-CSF each. The first immunization consisting of 20 µg was administered about three weeks after the primary immunization, and the second 20 µg boost was administered about one week later.

About five and one half weeks after the second 20 μg boost, a final immunization was conducted, consisting of administering 10 μg of rM-CSF intravenously. Three days later, spleens from immunized mice were removed and the splenocytes fused to a murine myeloma cell line.

The fusion procedure that was followed is described by Kohler & Milstein, 1975, Nature, 256:495, as modified by Fendly et al., in Hybridoma, 6:359 (1987). Briefly, mice were sacrificed and splenocytes teased from immunized spleens, and washed in serum free Dulbecco's Modified Eagles medium. Similarly, SP 2/0Ag14 myeloma cells were washed, and combined with the splenocytes in a 5:1 ratio of spleen cells to myeloma cells. The cell mixture was pelleted, media removed and fusion affected by the addition of 1.0 ml of 40% (v/v) solution of polyethylene glycol 1500 by dropwise addition over 60 seconds at room temperature, followed by a 60-second incubation at 37°C. To the cell suspension with gentle agitation was added 9 ml of Dulbecco's Modified Eagles medium over 5 minutes. Cell clumps in the mixture were gently resuspended, the cells washed to remove any residual PEG and plated at about 2 x 10^5 cells/well in Dulbecco's Modified Eagles medium supplemented with 20% fetal calf serum. After 24 hours, the cells were fed a 2x solution of hypoxanthine and azaserine selection medium. The cells were plated in a total of 15.5 microtiter plates, which corresponded to 1488 wells. About 2.4 weeks later, 684 wells exhibited good cell growth, and these were screened for antibody to M-CSF. Several neutralizing antibodies were identified such as those secreted by hybridomas 382-5H4, 382-3F1, and 382-4B5 or their subclones. These antibodies were purified using standard methods, and may be used below for the treatment of sepsis.

The preferred IL-6 antibody, 8M70, was generated essentially using the procedures to generate the M-CSF antibody with the following differences. Recombinant IL-6 was used as the immunogen, and it was produced using the procedures described by Brakenhoff et al., Journal of Immunology, 1987, vol. 139, page 4116. Eight separate fusions were conducted before the antibody was identified using RIA screening techniques. Using standard biochemical methods, it was determined to have a Kd of about 10^{-11}.

The hybridoma cell line, 8M70, also termed CLB-IL-6-8, was cultured in 1 liter roller-bottles in IMDM media supplemented with 2% fetal calf serum, 50 uM 2-mercaptoethanol, and penicillin and streptomycin. The cells were grown to a density of about 10^6/ml, and a week later the supernatants were collected, and concentrated using a hollow fiber device. To purify the antibody on a protein A column (Pharmacia) solid NaCl was added to the concentrate to a final concentration of 3 M. This solution was diluted 1:1 with a solution consisting of 3 M

NaCl and 1.5 M glycine, pH 8.9. The protein A column was equilibrated with the latter buffer, and the concentrate added to the column, the column washed, and antibody eluted off the column with 100 mM sodium citrate buffer, pH 6.0. Those peaks containing antibody were pooled, dialyzed against phosphate buffered saline, and stored until used.

B. Human Hybridomas/Human Monoclonal Antibody

Peripheral blood lymphocytes are isolated from septic patients, and then infected with Epstein-Barr virus and the infected lymphocytes immortalized by fusion to a selectable myeloma cell line, and the hybrid cell lines so generated isolated and characterized as to antibody production. More specifically, mononuclear cells are separated on Ficoll-hypaque (Pharmacia), and monocytes depleted from the mixture by adherence to plastic. Standard laboratory techniques are utilized to effect these procedures. Next, nonadherent cells are enriched for antibody producers by antigen-specific panning. Panning is a technique generally known in the art, and involves incubation of a population of antibody secreting cells on a plastic surface coated with the appropriate antigen, in this instance IL-6, M-CSF or peptide immunogens derived from these molecules, and produced as described in Example I. Those cells that express antibody on their surface bind antigen, and consequently adhere to the plastic surface, whereas cells that do not express cell surface antibody, do not adhere and can be removed by washing. Thus, specific antibody secreting cells are enriched for by this technique.

More specifically, 6-well plates (Costar) are coated with 1-20 μg of IL-6, M-CSF or peptide immunogens per well in phosphate buffered saline at 4°C overnight. The wells are blocked after the overnight incubation period with phosphate buffered saline containing 1% bovine serum albumin for at least 1 hour at 4°C, and subsequently washed with phosphate buffered saline/BSA. Next, 10^7 lymphocytes in 1 ml of PBS/BSA are added to each well of the six well plates. The lymphocytes are allowed to incubate on the plates for 70 minutes, after which any nonadherent cells are removed by aspiration. The adherent cells are incubated with cell culture medium (IMDM, Sigma Chemical Co., St. Louis, Missouri) containing 10% fetal calf serum.

The adherent cells are subjected to Epstein-Barr virus transformation by adding an equal amount of culture media obtained from growing the Epstein-Barr virus infected marmoset cell line, B95-8, or similar cell line, and thus containing the virus, to media bathing the adherent cells. The cells are cultured in this environment at 37°C for 3 hours, and in this way the lymphocytes in the adherent cell population are subjected to Epstein-Barr infection. Following the infection period, the cells are washed and

plated onto 96 well microtitre plates at a density of about 10^4 - 10^5 cells/well in IMDM medium, plus 10% fetal calf serum, and 30% conditioned medium. The latter is derived from a lymphoblastoid cell line, preferably JW5. The medium also contains 5 x 10^{-5} M 2-mercaptoethanol, 50 µg/ml gentamycin sulfate (Sigma), and 600 ng/ml cyclosporine A (Sandimmune, Sandoz, Basel, Switzerland).

After about 14 to 21 days of incubation, cell culture supernatants are combined and screened for the desired antibody binding activity as described below. Positive hybridomas are subcultured at low density, retested for activity, and grown up and fused to the cell line F3B6 using polyethylene glycol and the plate fusion technique known in the art. The latter technique is described by Larrick, J.W., 1985, in Human Hybridomas and Monoclonal Antibodies, E.G. Engleman, S.K.H. Foung, J.W., Larrick, and A.A. Raubitschek, Editors, Plenum Press, New York, page 446. F3B6 is a heteromyeloma cell line that is sensitive to growth in media containing 100 µM hypoxanthine, 5 µg/ml azaserine and 5 µM ouabain. It is on deposit with the American Type Culture Collection with accession no. HB8785. Finally, the resulting hybrids are again screened to insure that they produce the desired antibody.

Example III

IL-6 Antibody for the Treatment of Sepsis

The effectiveness of the IL-6 antibody, 8M70, in a baboon sepsis model system was tested essentially as described by Taylor, et al., 1987, J. of Clinical Inv., 79:918, and by Taylor, et al., 1988, Circulatory Shock, 26:227. Briefly, this consisted of first measuring IL-6 levels in baboon plasma in response to a lethal or sublethal dose of E. coli., and secondly, determining if IL-6 antibody was effective in treating sepsis by preventing the death, or prolonging the lives of septic animals. A lethal or sublethal dose of E. coli consisted of approximately 4 x 10^{10} and 0.4 x 10^{10} organisms, respectively.

Figure 1 shows that after administration of a lethal dose of E. coli, IL-6 levels start to increase after 1 hour, at which time it is about 1,500 pg/ml, and continue to increase for at least up to 6 hours to about 9,000 pg/ml. In contrast, Figure 1 also shows that there is little perceptible increase in IL-6 after administration of a sublethal dose of E. coli. Baboons that receive a lethal dose of E. coli invariably die within 16-32 hours. Taylor, et al., 1987, J. of Clinical Inv., 79:918, and by Taylor, et al., 1988, Circulatory Shock, 26:227.

The effectiveness of the IL-6 monoclonal, 8M70, in preventing the death or prolonging the life of baboons was tested using two administration routines wherein the antibody was delivered in physiological saline. In the first, 5.9 mg of antibody per kg

of body weight was administered in three separate doses at 24, 22, and 21 hours before a lethal challenge of bacteria. Alternatively, 5.0 mg of antibody per kg of body weight was administered in a single dose simultaneously with the bacterial challenge. Figure 2 shows that in both instances IL-6 monoclonal antibody considerably extends the lifetime of the baboons that received the multiple or single dose of antibody and survived for 48, and 60 hours, respectively. Recall that baboons that receive a lethal dose of E. coli invariably die within 16-32 hours.

Figure 3 shows the effect of IL-6 antibody given simultaneously with a lethal dose of bacteria on various physiological parameters including white blood cells (WBC) hematocrit (HCT), platelet (Plat) and fibrinogen (Fibr) levels, and mean systemic arterial pressure (MSAP). With the exception of MSAP, the antibody had little effect. The extent of decrease of the MSAP was about half that observed for control animals, and thus may reflect the life-prolonging activity of IL-6 antibody.

Example IV

M-CSF Antibody for the Treatment of Sepsis

The effect of M-CSF antibody for the treatment of sepsis can be determined by first measuring the levels of M-CSF in baboons administered a lethal dose of E. coli., and secondly, showing that blocking or reducing the increase in M-CSF with antibody prevents the death, or extends the lifetime of the animals. The baboon was administered E. coli as described in the preceding example, and M-CSF was measured using a RIA assay as described in U.S. Patent No. 4,847,201, or by Halenbeck et al., Journal of Biotechnology, 1988, vol. 8, page 45.

Figure 4 shows M-CSF plasma levels in a baboon treated with a lethal dose of E. coli. M-CSF was about 25 ng/ml after 1.3 hours, and increased to about 60 ng/ml after 4 hours. Thereafter, from 4 hours to 16 hours, the level fell to about 45 ng/ml.

The effectiveness of M-CSF antibody would be demonstrated as described in the preceding example by administering the antibody prior to, or simultaneous with the bacteria. A dose of 5.0-15 mg per kg of animal body weight, with 15 mg per kg preferred, would be effective in extending the lifespan of treated animals when administered simultaneously with the bacteria. However, it will be appreciated that a fully protective dosing regime can be determined empirically by those skilled in the art, and can consists of multiple doses of antibody.

Example V

Treatment of Sepsis in Humans with Antibody to IL-6 and/or M-CSF

Purified 8M70 antibody to IL-6, and M-CSF antibody (i.e. antibody secreted by hybridomas 382-5H4, 382-3F1, and 382-4B5 or their subclones are administered alone, in combination, or sequentially to human patients for the therapeutic or prophylactic treatment of sepsis. Prophylactically the dose would be given just prior to surgery, and repeated at least once thereafter. Therapeutically the dose would be given every 24-48 hours until remission of the disease is apparent. The initial therapeutic dose would be 5-15 mg per kilogram of patient body weight, and then reduced to 5-10 mg per kilogram.

Variations of the above embodiments will be readily apparent to those of ordinary skill in the art without departing from the scope of the present invention, as described in the following claims.

WE CLAIM:

1. A method for treating sepsis in an organism comprising administering to said organism a composition comprising effective amounts of IL-6 antibody and M-CSF antibody.

2. A method as described in claim 1, wherein said antibody to IL-6 is selected from the group consisting of polyclonal, monoclonal, single chain, bispecific, or recombinant antibody.

3. A method as described in claim 1, wherein said antibody to M-CSF is selected from the group consisting of polyclonal, monoclonal, single chain, bispecific, or recombinant antibody.

4. A method as described in claim 2, wherein said antibody to IL-6 comprises monoclonal antibody.

5. A method as described in claim 3, wherein said antibody to M-CSF comprises monoclonal antibody.

6. A method as described in claim 4, wherein said IL-6 antibody comprises human or humanized antibody.

7. A method as described in claim 5, wherein said M-CSF antibody comprises human or humanized antibody.

8. A method for treating sepsis in an organism comprising administering to said organism a composition comprising an effective amount of IL-6 antibody.

9. A method as described in claim 8, wherein said antibody to IL-6 is selected from the group consisting of polyclonal, monoclonal, single chain, bispecific, or recombinant antibody.

10. A method as described in claim 9, wherein said antibody to IL-6 comprises monoclonal antibody.

11. A method as described in claim 10, wherein said IL-6 antibody comprises human or humanized antibody.

12. A method for treating sepsis in an organism comprising administering to said organism a composition comprising an effective amount of M-CSF antibody.

13. A method as described in claim 12, wherein said antibody to M-CSF is selected from the group consisting of polyclonal, monoclonal, single chain, bispecific, or recombinant antibody.

14. A method as described in claim 13, wherein said antibody to M-CSF comprises monoclonal antibody.

15. A method as described in claim 14, wherein said M-CSF antibody comprises human or humanized antibody.

16. A composition useful for the prophylactic or therapeutic treatment of sepsis comprising an effective amount of antibody to M-CSF and IL-6

17. A composition as described in claim 16, wherein said antibody is selected from the group consisting of polyclonal, monoclonal, single chain, bispecific, or recombinant antibody.

18. A composition as described in claim 17, wherein said antibody comprises polyclonal antibody.

19. A composition as described in claim 17, wherein said antibody comprises monoclonal antibody.

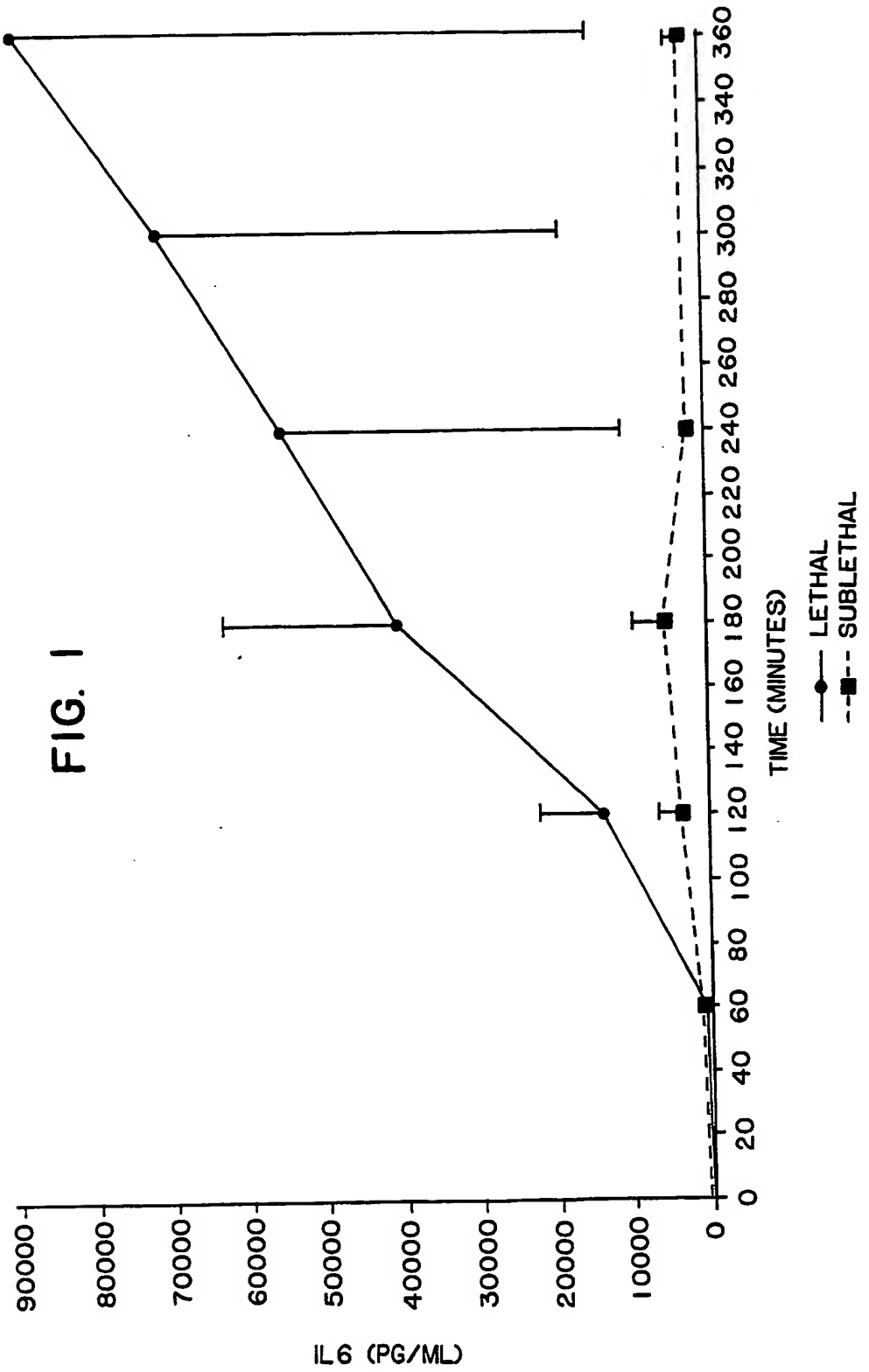

IL6 (PG/ML)
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360
TIME (MINUTES)
LETHAL
SUBLETHAL

FIG. 1

FIG. 2

IL-6

ANIMAL #	DOSE (mg/kg)	SCHEDULE * (HRS)	E.COLI/DOSE	RESULTS
1.	5.9	T-24 -22 -21	LETHAL	SURVIVED > 36 HRS (48 HRS)
2.	5.0	T_0	LETHAL	SURVIVED > 36 HRS (60 HRS)

* T_0 IS THE START OF E. COLI INFUSION.

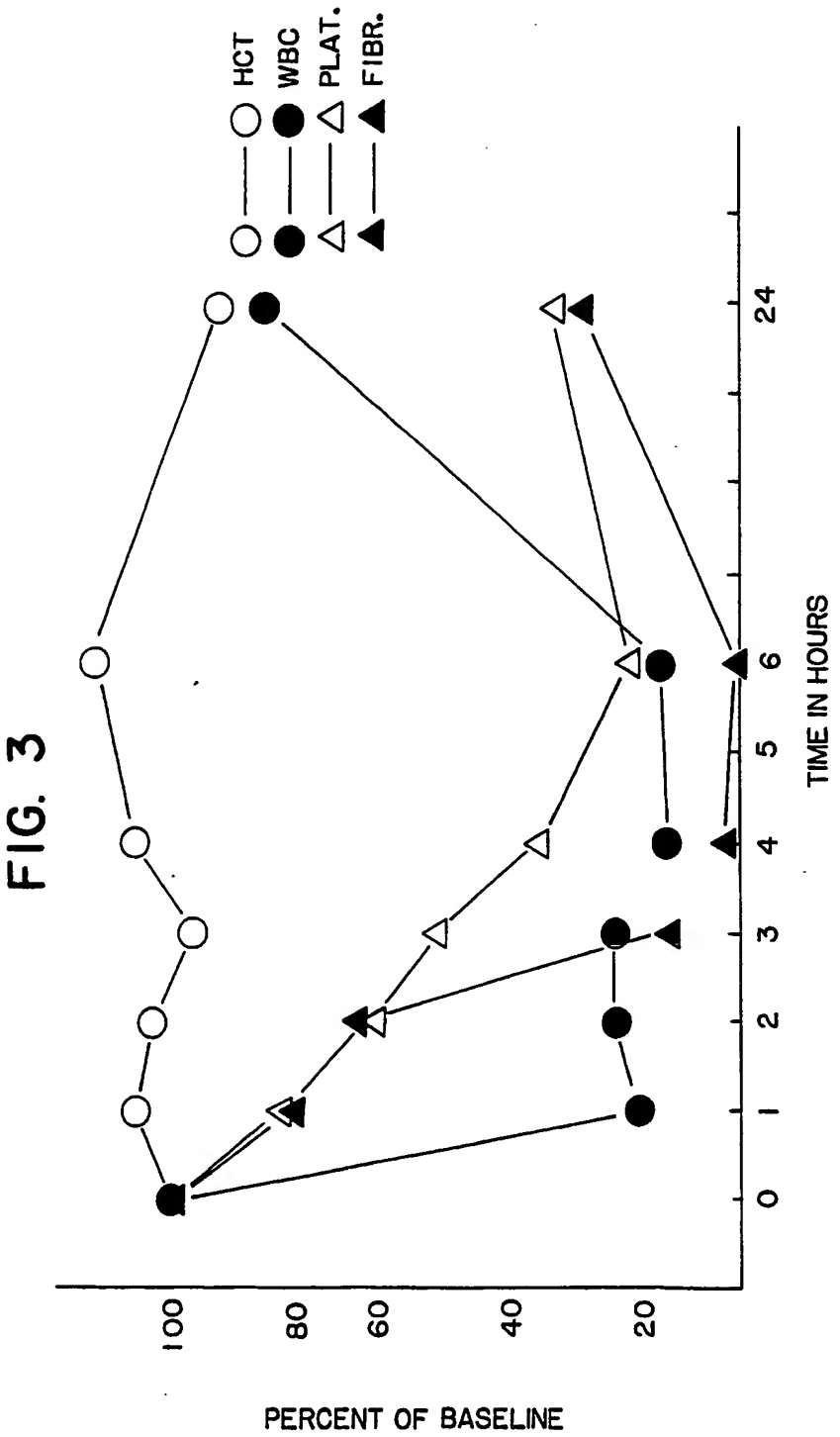
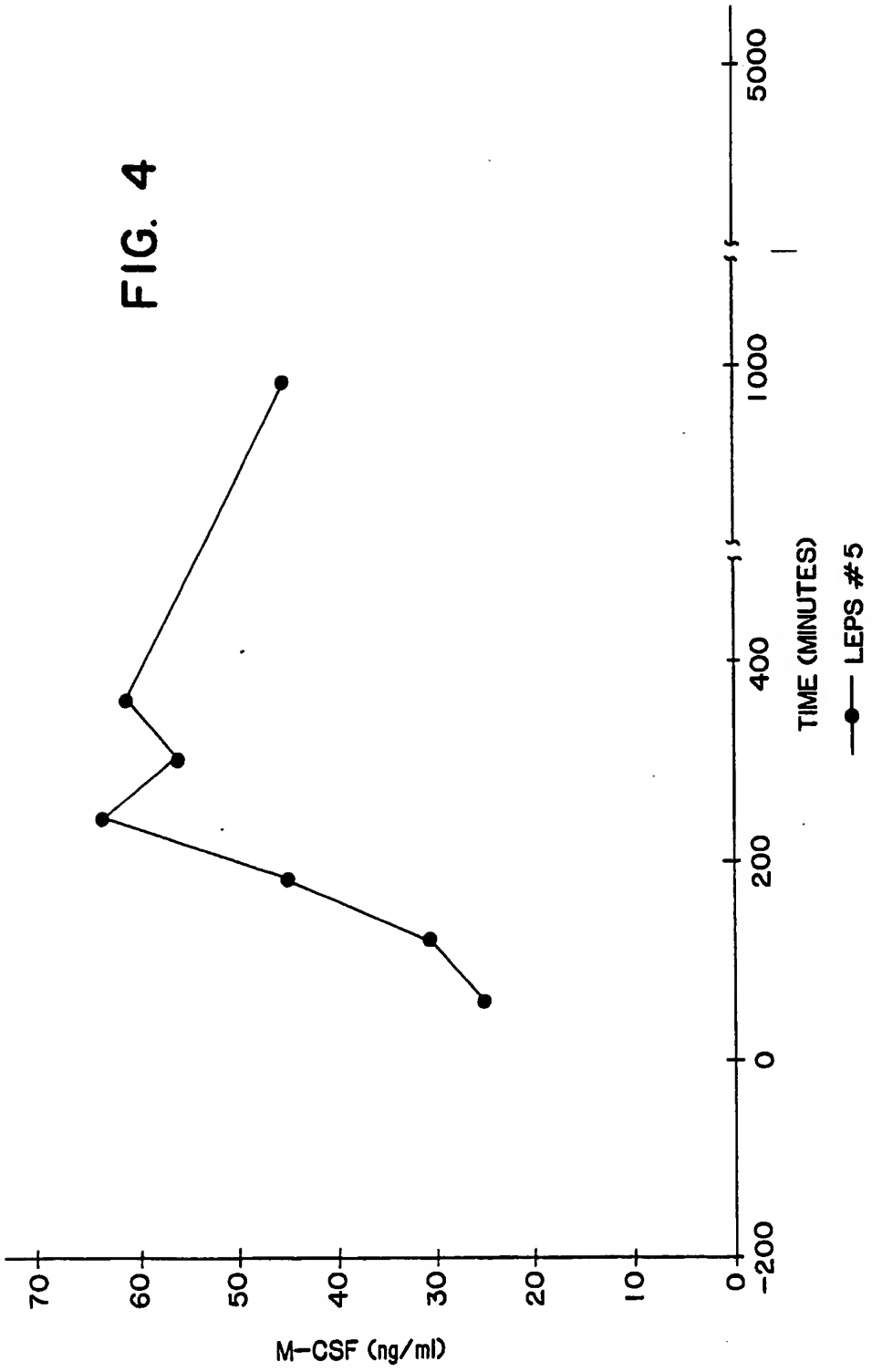

HCT
WBC
PLAT.
FIBR.
0
1
2
3
4
5
6
24
TIME IN HOURS
100
80
60
40
20
PERCENT OF BASELINE

FIG. 3

FIG. 4


M-CSF (ng/ml)
70
60
50
40
30
20
10
0
-200
0
200
400
1000
5000
TIME (MINUTES)
LEPS #5

INTERNATIONAL SEARCH REPORT

PCT/US 90/07411

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 39/395, C 12 P 21/08, C 07 K 15/28

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 61 K 39/00,C 12 P 21/00,C 07 K 15/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	EP, A2, 0 265 384 (SANDOZ-PATENT-GMBH) 27 April 1988 (27.04.88), see abstract. --	16-19
A	WO, A2, 89/03 846 (SCHERING BIOTECH CORPORATION) 05 May 1989 (05.05.89), see abstract. --	16-19
A	EP, A2, 0 111 344 (SLOAN-KETTERING INSTITUT FOR CANCER RESEARCH) 20 June 1984 (20.06.84), see abstract. --	16-19
A	EP, A2, 0 267 611 (OTSUKA PHARMACEUTICAL CO.) 18 May 1988 (18.05.88), see abstract. ----	16-19

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12 March 1991 (12.03.91)	10. 05. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Alfredo Prelm

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION 'TINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 1-15 because they relate to subject matter not required to be searched by this Authority, namely:

Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods. see PCT rule 39.1 (iv)

2. [ ] Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers _____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANHANG
zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

ANNEX
to the International Search Report to the International Patent Application No.

ANNEXE
au rapport de recherche international relatif á la demande de brevet international n°

SA43637.8 PCT/EP 90/07411

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

La présente annexe indique les membres de la famille de brevets relatifs aux documents de brevets cités dans le rapport de recherche international visée ci-dessus. Les reseignements fournis sont donnés á titre indicatif et n'engagent pas la responsibilité de l'Office.

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
EP-A2- 265384	27-04-88	EP-A3- 265384	28-09-88
		GB-A0- 8624899	19-11-86
		JP-A2-63126898	30-05-88
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		CN-A - 1032816	10-05-89
		DK-A - 1018/90	25-04-90
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		EP-A3- 314402	07-06-89
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		DK-A - 5903/87	14-05-88
		EP-A3- 267611	21-09-88
		KR-B1- 9004420	25-06-90
		JP-A2-63258595	26-10-88